REMARKS

Status of the Claims

Claims 1-2, 4-5, and 7-9 are amended and new claims 13-32 are added and should be part of the elected group. Each new claim is supported in the specification as-filed. Claims 6 and 10-12 are withdrawn. Upon entry of this Amendment, claims 1-32 are pending, and elected claims 1-5, 7-9, and 13-32 should be examined. No new matter has been added.

Initialed PTO 1449

Please re-send with the next Office action the initialed PTO 1449 from the previously filed IDS.

Objections to the Specification

The specification has been objected to because it is not in compliance with the Sequence Rules and Regulations of 37 CFR 1.821-1.825. See Office Action mailed February 27, 2003, page 2. Applicants have amended the specification to indicate the corresponding SEQ ID NOs referenced in Figures 1, 2A, and 2B. A substitute computer readable form with all the files of the original that were not amended is also filed herewith. Applicants respectfully request reconsideration and withdrawal of the rejection.

Claim Objections

Claims 1, 5, and 8 are objected to on the grounds that the claims embrace non-elected subject matter. The present version of the claims avoids this issue and thus the objection should be withdrawn.

Rejections- 35 U.S.C. § 101 (Non-statutory Subject Matter)

Claims 1, 4, and 8 are rejected under 35 U.S.C. §101 for allegedly embracing non-statutory matter. The present version of the claims avoids this issue. Therefore, Applicants respectfully request withdrawal of the rejection.

Rejections- 35 U.S.C. § 101 (Utility)

Claims 1-5 and 7-9 are rejected under 35 U.S.C. §101 for alleged lack of a specific and substantial asserted utility or a well-established utility. Applicants respectfully traverse this rejection.

According to the PTO, the claimed invention lacks a specific, substantial, and credible utility, or alternatively, a well-established utility. Office Action, pages 3-7. For this type of rejection, because utilities are asserted in the application, the rejection must be supported by explanation and supporting evidence. MPEP § 2107. Otherwise a rejection is improper and should be withdrawn.

Such is the case here. For example, as the Examiner even admits, the specification teaches that the amino acid sequence encoding Delta1 is a "novel cytokine receptor." See Office Action, page 4. The specification discloses that the gene encoding the protein was cloned from lymphocyte, and furthermore, northern analysis revealed that it was expressed in the heart, lung, liver, and spleen, as well as in myeloid and lymphoid cell lines such as Ba/F3, DA-1 and CTLL2. The specification further discloses that the membrane proximal region of human EPOR can be replaced with that region from the inventive protein and the engineered protein can activate JAK2. See specification, page 5, lines 15-25. Therefore, the receptor-like protein of the present invention is described as playing a regulatory role in immunity and haematopoiesis, which, of course, is specific.

Moreover, the present invention is described as making possible other specific utilities, namely, tools for purifying and/or cloning factors related to the functions of the immune system. For example, the specification discloses that the transcript encoding Delta 1 has been identified in the heart, brain, spleen, lung, kidney and testis, but not in skeletal muscle. See specification, Figure 4, and page 25, lines 15-18. Thus, Delta 1 is described as a tissue-specific marker, a specific utility. Additionally, the precise chromosomal location of Delta 1 has been determined. See specification, Example 7. Thus, the guidance from the present specification makes it possible to use Delta 1 as a genetic marker for many purposes,

including gene mapping, cloning, and chromosomal aberration tests, each of which, of course, are specific utilities.

Furthermore, not only is this utility specific, but this utility is also substantial, i.e., defines a real world use, for reasons that should need no explanation. Cf. MPEP 2107.01 I. Nevertheless, the present invention is described as having substantial utility as a drug candidate for immune system-related diseases. It is known that the ligand of the protein of the invention is the thymic stromal lymphopoietin (TSLP), which facilitates B lymphopoiesis and stimulates thymocytes and mature T cells (Pandey, A. et al., *Nature immunol.* 1, 59-64, 2000; Park, L.S. et al., *J. Exp. Medicine* 192: 659-669, 2000). (Enclosed for consideration). Thus, the inventive protein seems to impart a regulatory role in immunity and hematopoiesis and therefore, the protein is described as making it possible to screen for compounds for treating immune system-related diseases, i.e., a substantial utility. Additionally, the inventive protein is described as making possible therapies, such as gene therapy, for a variety of infectious diseases and autoimmune diseases. See specification, page 5, lines 24-34. Therapies, surely, are substantial.

As discussed above, the subject matter embraced by the elected claims are described as having specific, substantial, and credible utility. Neither evidence nor explanation shows otherwise. Therefore, the rejection is improper and should be withdrawn.

Rejections- 35 U.S.C. § 112, first paragraph (enablement)

Claims 1-5 and 7-9 are rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. Office Action, pages 7-9. According to the PTO, since the claimed invention is not supported by specific or substantial asserted utility, the specification does not enable any person skilled in the art to use the invention. Furthermore, the PTO alleges that the specification is not enabled on the grounds that "the specification does not teach how to make any variant of the instant invention, and provides no reliable assay to evaluate the function of any modified polypeptide." Office Action, page 7. Applicants respectfully traverse this rejection.

As discussed above, the present invention finds specific and substantial utility. Accordingly, this grounds for rejection is improper and should be withdrawn.

The present version of the claims are believed to avoid the other concerns addressed in the Office Action. The present version of claims 1-5 and 7-9 are believed supported by an enabling disclosure, and thus, the rejections should be withdrawn.

Additionally, claims are assumed enabled. MPEP § 2164.04. Indeed, when challenging a claim's enablement, the PTO must not only explain why it doubts the claim's presumptively enabling disclosure but also cite supporting evidence for its assertion. Id. "Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." Id. (citation omitted).

The PTO's explanation must further include specific technical reasons that cast doubt on the claim's enablement. Id. Yet, in the present rejection, the PTO's explanation consists of generalized, i.e., nonspecific, findings. For example, characterizing the predictability of the art, the PTO urged that "the effects of these changes are largely unpredictable...." Office action, p. 8. Since, according to the PTO, most, but not all, changes are unpredictable, the PTO admitted some changes are predictable. Since no uses of the present invention were discussed by the PTO, no explanation supports the PTO's assertion about how "to use" for all its uses in the present specification.

Furthermore, the PTO never cited sufficient evidence to support its assertions. All factual findings that are material to patentability must be supported by substantial evidence. In re Zurko, 258 F.3d 1379, 1386 (Fed. Cir. 2001). In this rejection, however, the PTO cited virtually no evidence to support its findings.

And contrary to the PTO's position, the present invention indeed discloses methods for making variants of the instant invention. In particular, the specification discloses "proteins functionally equivalent to SEQ ID NO: 2 preferably have features of a type I cytokine receptor. Such features can be exemplified by regions such as box 1 and box 2." See specification, page 6, lines 1-13. Moreover, the application discloses "both boxes are important for intracellular signal transduction and, in particular, box 1 is considered important for interaction of Jak." Since the specification clearly indicates conserved regions associated with the inventive protein, one of skill in the art would know how to make a variant or mutant protein having an equivalent function. Moreover, the specification discloses several methods for introducing mutations into proteins. For example, site-directed mutagenesis is described on page 6, lines 24-25. Finally, the specification provides a means for assaying activity of a mutant or variant protein of the inventive protein, as indicated by the ability to activate JAK2. See page 5, lines 20-25 and Example 7.

Neither evidence nor explanation of record casts doubts on the present claim's enablement. Thus, this rejection is improper and should be withdrawn.

Rejections- 35 U.S.C. § 112, first paragraph (written description)

Claims 1, 4, and 7-8 are rejected under 35 USC § 112, first paragraph, for alleged lack of written description. Office Action, pages 9-11. Specifically, the PTO asserts "the specification provides adequate written description for SEQ ID NOs: 1 and 2, but no variants." Moreover, the PTO claims that in the absence of a recitation of clear hybridization conditions, a nucleic acid probe will hybridize with unrelated DNA sequences, corresponding sequences from other sequences, mutated sequences, allelic variants, and splice variants. Office Action, page 10.

The present version of the claims are believed to avoid the concerns addressed in the Office Action. As the present version of the claims meet the requirements for written description under 35 U.S.C. § 112, Applicants respectfully request reconsideration and withdrawal of the rejection.

Furthermore, the rejection concerned as-filed claims. Thus, there is a strong presumption that an the specification as-filed provides an adequate written description. MPEP § 2163 I. A. Furthermore, the evidence and explanation of record fail to prima facie show that applicant was not in possession of each claim as-filed, as well as the present version of the claims as-amended.

Specifically, the specification discloses specific hybridization conditions for isolating DNA encoding a functionally equivalent protein of the present invention. In particular, the PTO is directed to page 8 of the specification, lines 19-33, wherein stringent hybridization conditions are disclosed Furthermore, the specification discloses that the a gene encoding a functionally equivalent protein may be identified by PCR using a portion of the DNA encoding the protein (e.g. SEQ ID NO: 1) as a primer. See page 8, lines 31-34. Therefore, the specification discloses at least two independent methods for obtaining a functionally equivalent protein of the present invention. For these reasons alone, the rejection is improper and should be withdrawn.

Additionally, the specification clearly indicates "significant cross-hybridizing does not occur with DNAs encoding other proteins under normal hybridization conditions, preferably under stringent hybridization conditions. Such nucleotides include probes, primers, and nucleotides or nucleotide derivatives (for example, antisense oligonucleotides or ribozymes), all of which can hybridize specifically with the DNA encoding the protein of the present invention, or its complementary strand." See specification, page 14, lines 19-26. For these reasons too, the rejection is improper and should be withdrawn.

Rejections- 35 U.S.C. § 102(b)

Claims 1, 4, and 7-8 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Noguchi et al. (Blood, 1991, IDS A7, Paper No. 9). According to the Examiner, "[i]n the absence of a recitation of clear hybridization conditions, any polynucleotide sequence will hybridize to SEQ ID NO: 1." See Office Action, page 12. Applicants respectfully traverse this rejection.

A reference anticipates a claim only if that reference describes each and every element of the claim. MPEP § 2131. If even just one element is absent from the reference's particular disclosure, that disclosure neither describes nor anticipates the claim. Id. Such is the case in this rejection.

Even if the PTO's position were accurate, Noguchi et al. does not describe "a DNA encoding a protein consisting of an amino acid sequence with 70% or higher homology to SEQ ID NO: 2." As evidenced by BLASTP analysis (enclosed for consideration), the homology between the protein of the invention (Delta1) and the protein of Noguchi et al. (erythropoietin receptor) is not 70% or higher. Therefore, Noguchi et al. never describe "a DNA encoding a protein consisting of an amino acid sequence with 70% or higher homology to SEQ ID NO: 2." Accordingly, Noguchi et al. does not anticipate claims 1, 4, and 7-8. Applicants respectfully request reconsideration and withdrawal of the rejections.

CONCLUSION

As the above-presented amendments and remarks address and avoid all of the rejections presented by the Examiner, withdrawal of the rejections and allowance of the claims are respectfully requested. No new matter has been added.

If there are any questions concerning this application, the Examiner is courteously invited to contact the undersigned counsel.

Respectfully submitted,

Date 5/27/03 By _____

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 19-0741 for any such fees; and applicant(s) hereby petition for any needed extension of time.

Enclosures:

Pandey, A. et al., *Nature immunol.* 1, 59-64 (2000)
Park, L.S. et al., *J. Exp. Medicine* 192: 659-669 (2000)
BLASTP 2 Sequences Results

Blast 2 Sequences results

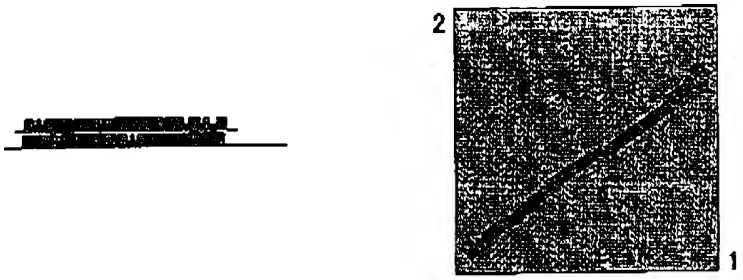
NCBI PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.6 [Apr-09-2003]

Matrix BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 10.000 wordsize: 3 Filter ☐

Sequence 1 gi 7077158 cytokine receptor delta1 [Mus musculus] **Length** 359 (1 .. 359)

Sequence 2 gi 4503591 erythropoietin receptor precursor [Homo sapiens] **Length** 508 (1 .. 508)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 51.6 bits (122), Expect = 3e-05
Identities = 95/371 (25%), Positives = 129/371 (34%), Gaps = 71/371 (19%)

```
Query:                          20  AAAVTSRGDVTVVCHD--LETVEVTW-------GSGPDHHGANLSLEFR-YGTGALQPCPR 70
                                    AA + +RG   ++C    LE +   W       G GP ++  +  LE   +    L   P
Sbjct:                          39  AALLAARGPEELLCFTERLEDLVCFWEEAASAGVGPGNYSFSYQLEDEPWKLCRLHQAPT 98
erythropoietin receptor         39  ************************************************************

Query:                          71  YFLSGAGVTSGCILPAARAGL---LELALRDGGGAMVF-KARQRASAWLKPRPPWNVTLL 126
                                      +   V   C LP A        LEL +    GA  + +          L   P   V  L
Sbjct:                          99  --ARGAVRFWGSLPTADTSSFVPLELRVTAASGAPRYHRVIHINEVVLLDAPVGLVARL 155
erythropoietin receptor         99    ************************************************************
Fibronectin type 3 domain      146                                                          **********

Query:                         127  WTPDGDVTVSW-------PAHSYLGLDYEVQHRESNDDEDAWQTTSGPCCDLTVGGLDPAR 180
                                      G V + W         P  S++  + +V              +   G   +  +  L
Sbjct:                         156  ADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRT-ECVLSNLRGRT 214
Fibronectin type 3 domain      156  ************************************************** **********
erythropoietin receptor        156  ************************************************** **********

Query:                         181  CYDFRVRASPRAAHYGLEAQPSEWTAVTRLSGAASAASCTASPAPSPALAPPLLPLGCGL 240
                                     Y F VRA           A+PS          G  SA S   S       L P +L L   L
Sbjct:                         215  RYTFAVRARM-------AEPS-------FGGFWSAWSEPVSLLTPSDLDPLILTLSLIL 259
erythropoietin receptor        215  **********         ****        ********************************
Fibronectin type 3 domain      215  *********

Query:                         241  AALLTLSLLLAALRLRR-VKDALLPCVPDPSGSFPGLFEKHHGNFQAWIADAQATA---- 295
                                      +L L  +LA L  RR +K  + P +P P   F GLF  H GNFQ W+
Sbjct:                         260  VVILVLLTVLALLSHRRALKQKIWPGIPSPESEFEGLFTTHKGNFQLWLYQNDGCLWWSP 319
erythropoietin receptor        260  ************************************************************

Query:                         296  -------PPARTEEEDDLIHPKAKRVEP---EDGTSLCTV----------------PR- 327
                                          PPA  E   +      + VEP   ++G  L  V                    PR
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Appendix 3 (Blast Result)

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Sbjct:                  320 CTPFTEDPPASLEVLSERCWGTMQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDKWLLPRN 379
erythropoietin receptor 320 ************************************************************

Query:                  328 PPSFEPRGPGG 338
                            PPS +  GPGG
Sbjct:                  380 PPSEDLPGPGG 390
erythropoietin receptor 380 ***********

CPU time:     0.03 user secs.          0.00 sys. secs     0.03 total secs.

Lambda     K      H
   0.319    0.135    0.433

Gapped
Lambda     K      H
   0.267   0.0410    0.140


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Hits to DB: 2086
Number of Sequences: 0
Number of extensions: 195
Number of successful extensions: 1
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 359
length of database: 477,760,714
effective HSP length: 128
effective length of query: 231
effective length of database: 477,760,586
effective search space: 110362695366
effective search space used: 110362695366
T: 9
A: 40
X1: 16 ( 7.4 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 41 (21.7 bits)
S2: 75 (33.5 bits)
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Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin

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Signaling by type I cytokines involves the formation of receptor homodimers, heterodimers or higher order receptor oligomers. Here we report the cloning of a type I cytokine receptor subunit that is most closely related to the common cytokine receptor γ chain (γ_c). Binding and crosslinking experiments demonstrate that this protein is the receptor for a recently described interleukin 7 (IL-7)-like factor, thymic stromal lymphopoietin (TSLP). Binding of TSLP to the thymic stromal lymphopoietin receptor (TSLPR) is increased markedly in the presence of the IL-7 receptor α chain (IL-7Rα). IL-7Rα–expressing but not parental 32D cells proliferate in the presence of exogenous TSLP. Moreover, a combination of IL-7Rα and TSLPR is required for TSLP-dependent activation of a STAT5-dependent reporter construct. Thus it is shown that IL-7Rα is a component of both the IL-7 and TSLP receptors, which helps to explain why deletion of the gene that encodes IL-7Rα affects the lymphoid system more severely than deletion of the gene encoding IL-7 does. Cloning of TSLPR should facilitate an understanding of TSLP function and its signaling mechanism.

Cytokines regulate a variety of cellular responses including proliferation, differentiation and survival. There are several different classes of cytokines with varied structures. Type I cytokines form four α-helical bundle structures that exhibit an up-up-down-down topology[1–3]. They include many interleukins (IL), such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13 and IL-15, other hematologically active molecules such as granulocyte-macrophage colony stimulating factor (GM-CSF) erythropoietin, thrombopoietin, and other molecules such as growth hormone and prolactin. The receptors for these molecules are members of the cytokine receptor superfamily, also known as type I cytokine receptors[1–3].

Of all the cytokine receptor signaling systems, IL-2 and its receptor complex (IL-2R) is one of the best studied. IL-2 is a cytokine that plays a pivotal role in the regulation of T cell-mediated immune responses[4,5]. The IL-2R consists of three subunits: the IL-2 receptor α chain (IL-2Rα), IL-2 receptor β chain (IL-2Rβ) and the common γ chain (γ_c). The high-affinity IL-2R complex consists of all the three subunits, the intermediate-affinity complex is comprised of IL-2Rβ and γ_c, and the low-affinity receptor is comprised of IL-2Rα only[5–7]. The α subunit lacks any signaling capability but is required for binding to the ligand. Formation of intermediate- and high-affinity receptor complexes leads to heterodimerization of the cytoplasmic domains of IL-2Rβ and γ_c, which is required for IL-2 signaling[8,9]. Addition of IL-2 to responsive cells also leads to activation of the JAK family of cytoplasmic tyrosine kinases as well as STAT proteins—STAT3, STAT5A and STAT5B[4,10–15]. γ_c associates with JAK3 and is essential for signaling[16–32]. Mutations in the γ_c gene lead to X-linked severe combined immunodeficiency (XSCID) in humans and several lymphocyte abnormalities in mice[4,16]. In addition to IL-2, γ_c is a signaling component of the receptor complex for several ligands of the IL-2 subfamily—IL-4, IL-7, IL-9 and IL-15[10,17–23].

Here we report the isolation of a Type I cytokine receptor subunit that is most similar to murine γ_c. The open reading frame encodes a transmembrane receptor containing 370 amino acids (aa) with two potential N-linked glycosylation sites and a cytoplasmic domain of 104 aa including a single tyrosine residue. This receptor was unable to bind to several IL-2 subfamily cytokines including IL-2, IL-4, IL-7 and IL-15. The cytokine thymic stromal lymphopoeitin (TSLP) was originally identified as an activity from the conditioned medium of a thymic stromal cell line that supported the development of IgM$^+$ B cells from fetal liver hematopoietic progenitor cells and it was suggested that TSLP requires IL-7 receptor α (IL-7Rα) but not γ_c subunits for signaling[24,25]. Activities of TSLP overlap somewhat with those of IL-7: both facilitate B lymphopoiesis in cultures of fetal liver and bone marrow lymphocyte precursors, and both costimulate thymocytes and mature T cells[11,24]. The difference in biological activities which occurred despite a shared receptor (IL-7Rα) implied the presence of a distinct receptor subunit for TSLP. Here we demonstrate that the receptor subunit we identified is required for binding to TSLP and hence we named this protein TSLPR. This binding is also dependent upon the

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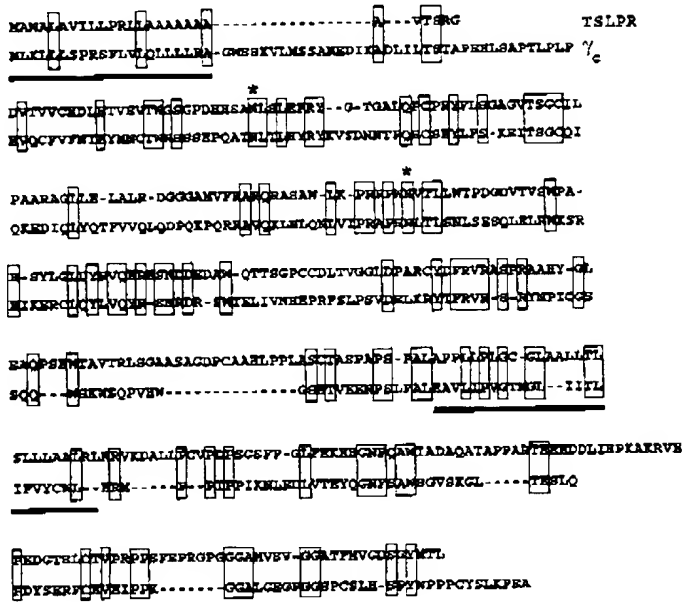

Figure 1. A ubiquitously expressed receptor subunit (TSLPR) shares homology with murine γ_c. An alignment of TSLPR with the murine γ_c is shown with identical residues boxed and the potential N-linked glycosylation sites marked with an asterisk (*). The signal peptides and transmembrane domains are underlined. The DNA sequence of TSLPR has been deposited with Genbank (accession number AF201963).

presence of IL-7Rα. Only when *IL7R* is transfected into 32D cells can TSLP replace the requirement for IL-3 for the growth of 32D cells. Finally, because activation of a STAT5-dependent reporter construct required the presence of both TSLPR and IL-7Rα in a reconstitution system we can demonstrate the involvement of TSLPR in signaling.

Results

Cloning of a Type 1 cytokine receptor subunit that is similar to γ_c

Given the importance of cytokine receptors, we sought to identify additional receptors of the cytokine receptor superfamily. To do this, we used the cytoplasmic domain of the erythropoietin receptor in a search using the basic local alignment search tool (BLAST) algorithm against the EST database (dbEST, searchable at the National Center for Biotechnology Information). Several overlapping murine ESTs (accession numbers AA018020, AA015052, AA008678) were found which encoded a type I cytokine receptor molecule that had significant similarity to γ_c, erythropoietin receptor and the IL-9 receptor α chain in its cytoplasmic domain. As none of the ESTs contained the entire open reading frame, a mouse embryo library was screened to obtain a full-length cDNA. The longest positive clone contained a 2 kb insert which was sequenced. The open reading frame encoded a type I transmembrane protein containing 370 aa, with two potential *N*-linked glycosylation sites and a cytoplasmic domain of 104 aa containing a single tyrosine residue. Murine γ_c is comprised of 369 aa with a short cytoplasmic domain of 86 aa containing two tyrosine residues[26-28]. An alignment of this newly identified γ_c-like Type 1 cytokine receptor molecule with murine γ_c showed 26% identity and 47% similarity at the protein level (**Fig. 1**). Its sequence is slightly atypical for type I cytokine receptors in that only one pair of cysteines is conserved and the usual WSXWS motif is replaced by WTAVT. The predicted molecular mass was 37 kD. Consistent with this finding, when a construct containing the entire open reading frame was transcribed and translated *in vitro*, a single species of approximately 40 kD was observed (**Fig. 2a**). A polyclonal antiserum raised against the extracellular domain specifically immunoprecipitated a broad band of approximately 50 kD in a pre-B cell line NAG8/7 (**Fig. 2b**). The fact that a larger protein was identified in NAG8/7 lysates compared to the *in vitro* translation product is consistent with *N*-linked carbohydrate addition sites in the extracellular domain.

Expression pattern of the γ_c-like receptor

Flow cytometric analysis of the transfected 293T cells or several hematopoietic cell lines (32D, BaF3 and WEHI-3) confirmed cell surface expression of this receptor (data not shown). To examine the tissue distribution of this receptor, we carried out northern blot analysis. As shown in **Fig. 2c**, transcripts were detected in all tissues tested, with highest levels of expression being found in liver, lung and testis, fol-

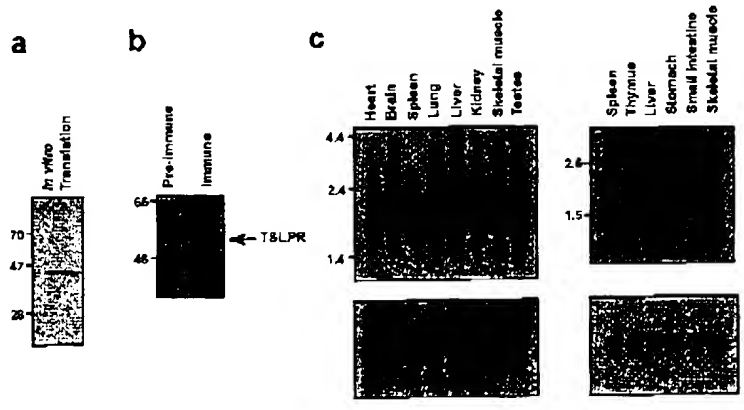

Figure 2. The receptor is ubiquitously expressed. (a) A cDNA construct encoding the receptor was transcribed and translated *in vitro* in the presence of ^{35}S labeled methionine and the product resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). An autoradiogram of the gel is shown with molecular mass markers in kD indicated on the left. (b) The growth factor-dependent NAG8/7 pre-B cell line was metabolically labeled with ^{35}S-methionine and cysteine and the lysates immunoprecipitated with either a rabbit polyclonal antibody directed against the extracellular domain of the novel receptor or pre-immune serum as indicated. (c) Mouse multiple tissue northern blots (left panels: Clontech, Palo Alto; right panels: Origene Technologies, Rockville) were probed with a *TSLPR* cDNA probe labeled with ^{32}P. RNA markers (in kb) are indicated on the left. The bottom panels show reprobing with a probe from *β-actin* to confirm equal loading.

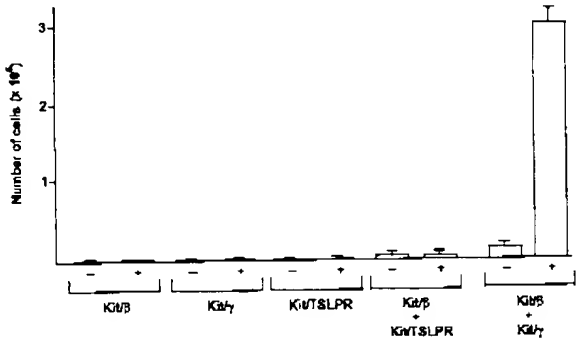

Figure 3. Homodimerization of TSLPR does not lead to proliferation. IL-2-dependent CTLL2 cell line was stably transfected with Kit–β alone, Kit–γ alone, Kit–TSLPR alone, Kit–β and Kit–TSLPR or Kit–β and Kit–γ. The cells were then deprived of IL-2, transferred into a 48-well dish and grown in the absence (–) or presence (+) of SCF. The number of viable cells was counted after 7 days.

lowed by spleen, thymus, brain, heart and skeletal muscle. Two transcripts of approximately 2 kb and 2.2 kb were detected in some tissues, whereas others contained only a single transcript. This broad tissue distribution contrasts to the relatively restricted lympho-hematopoietic pattern of expression seen with γ_c.

Homodimerization does not lead to proliferation

Because of its similarity to the erythropoietin receptor, which is activated by homodimerization, we reasoned that this Type 1 cytokine molecule might signal when induced to homodimerize. To test this hypothesis, a chimeric Kit–TSLPR construct was made with the extracellular and transmembrane domains derived the c-Kit receptor and the cytoplasmic domain from the novel receptor. IL-2-dependent CTLL2 cells stably expressing such a construct were used for this purpose. However, when IL-2 was replaced by stem cell factor (SCF)—the ligand for c-Kit—the cells were unable to grow, suggesting that simple homodimerization of the cytoplasmic domain of this novel receptor was insufficient to induce a proliferative signal. This is similar to what is observed with a c-Kit–γ_c chimeric molecule (Kit–γ) [8]. When Kit–TSLPR was cotransfected with a plasmid encoding a Kit–β chimera into CTLL2 cells, the cells were still unable to proliferate although cells coexpressing both Kit–β and Kit–γ proliferated well in response to SCF (**Fig. 3**). This suggested that the cytoplasmic domain of IL-2Rβ could not cooperate with the cytoplasmic domain of this novel γ_c-like receptor to initiate proliferation. Both of these results suggested that this receptor might oligomerize with some other receptor in order to signal.

TSLP binds to the γ_c-like Type 1 cytokine receptor subunit

Because this receptor was similar to γ_c, we tested its capacity to bind to some of the members of the IL-2 cytokine subfamily. ^{125}I-labeled IL-2, IL-4, IL-7 and IL-15 were used for binding and crosslinking experiments in 293T cells reconstituted with the cytokine-specific subunits (IL-2Rβ, IL-4Rα, IL-7Rα) in the presence of γ_c or the γ_c-like Type 1 cytokine receptor. None of these ligands exhibited crosslinking to the γ_c-like protein, even though they could be efficiently crosslinked to coexpressed γ_c (data not shown). These experiments suggested that this molecule is a receptor subunit which may bind to an unknown cytokine(s) or to a known cytokine(s) in conjunction with novel or untested subunit(s). As previously mentioned, TSLP is a cytokine whose biological activities overlap with those of IL-7. Although both cytokines are involved in supporting IgM$^+$ B cell development[25], promoting B cell lymphopoiesis, and can costimulate both thymocytes and mature T cells[24,25,28–31], TSLP is unique in its ability to promote B lymphopoiesis to the IgM$^-$ immature B cell stage, whereas IL-7 primarily facilitates production of IgM$^-$ pre-B cells[24,32]. Some of these overlapping biological activities of IL-7 and TSLP might be explained by data suggesting that TSLP signals via a receptor that contains IL-7Rα[24]. However, antibody-inhibition experiments indicated that TSLP did not require γ_c to exert its effects[24]. This led us to examine whether the γ_c-like receptor could be a subunit that bound TSLP in the presence of IL-7Rα.

We next used affinity labeling with ^{125}I-TSLP and disuccinimidyl suberate as a crosslinker. As shown in **Fig. 4a**, ^{125}I-TSLP bound to the heterodimer of murine TSLPR and murine IL-7Rα, but not to murine IL-7Rα alone. The upper band corresponds to crosslinked murine IL-7Rα and the lower band corresponds to murine TSLPR. ^{125}I-TSLP also bound to the heterodimer of human IL-7Rα and murine TSLPR. Because human IL-7Rα has a larger molecular mass than murine IL-7rα, we can determine which affinity-labeled band corresponds to IL-7Rα and TSLPR. Moreover, the expression and sizes of human IL-7Rα and murine IL-7Rα were confirmed by western blotting (data not shown). As shown in **Fig. 4a** and **b**, although the signal from direct lysates of cells transfected only with murine *Tslpr* is weak, there is a crosslinked TSLPR band after TSLPR immunoprecipitation, suggesting that TSLP exhibits weak binding to TSLPR alone. Next, we addressed whether murine IL-7 could compete with TSLP binding to cells expressing a combination of TSLPR and IL-7Rα. As shown in **Fig. 4c**, an excess of murine IL-7 inhibited the binding of TSLP to the TSLPR–IL-7Rα heterodimer. The affinity labeling experiments demonstrated the cooperativity of IL-7Rα and the newly identified γ_c-like receptor in binding TSLP. They also establish that IL-7 can compete for binding TSLP, which has implications for potential competition between these two cytokines *in vivo*.

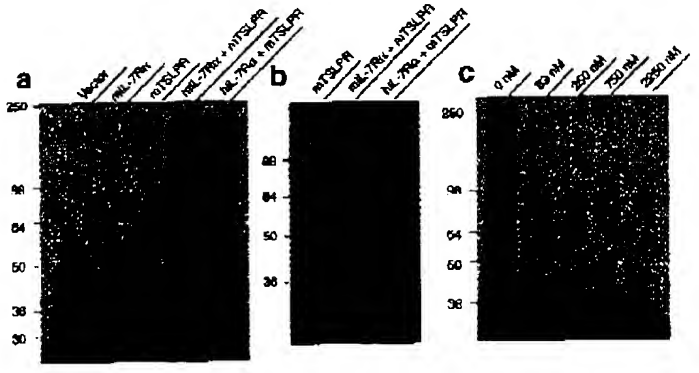

Figure 4. Affinity labeling of TSLPR and IL-7Rα using ^{125}I-TSLP. 5 nM ^{125}I-TSLP was added to 293T cells transfected with various receptor subunits as shown and crosslinked with disuccinimidyl suberate (DSS). After affinity labeling cells were either (a) lysed in lysis buffer and analysed by SDS-PAGE or (b) first immunoprecipitated with anti-Flag monoclonal M2 antibody (TSLPR is tagged with Flag at C-terminus) and then analysed by SDS-PAGE. In (c) lysates were analysed directly as in (a), except that cold competition was performed with increasing concentrations of unlabeled murine IL-7 as indicated. (prefix "m", murine; prefix "h", human.)

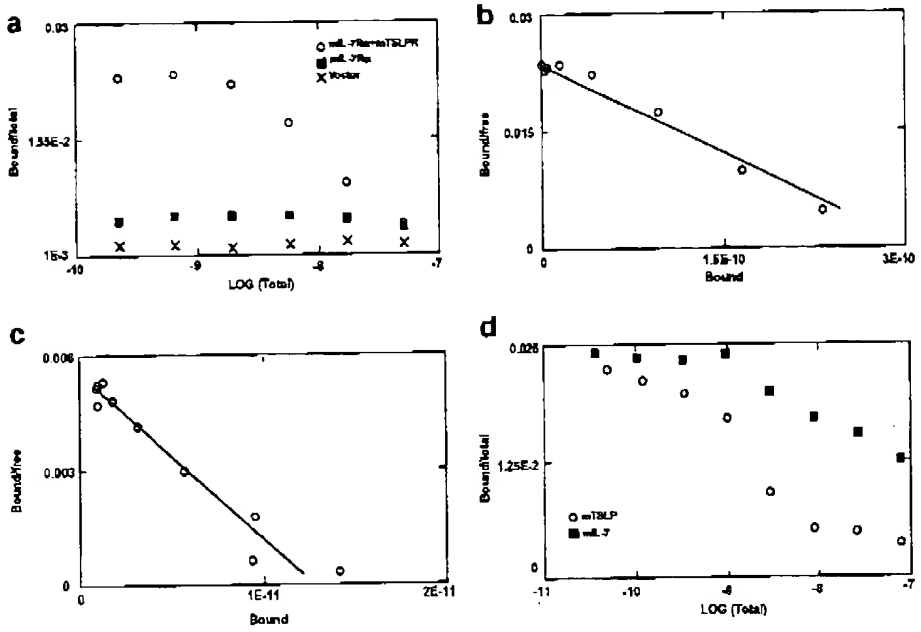

Figure 5. High affinity TSLP receptors—displacement binding experiment. A constant amount of ^{125}I-labeled TSLP was combined with varying amounts of unlabeled TSLP and added to transfected 293T cells. (a) Ligand displacement curve for 293T cells transfected with vector alone or the receptor subunits as shown. (b) Scatchard curve of TSLP binding to 293T cells transfected with *TSLPR* and IL-7Rα. (c) Scatchard curve of TSLP binding to NAG8/7 cells. (d) Ligand displacement curve for 293T cells transfected with *TSLPR* and IL-7Rα; ^{125}I-TSLP was bound and binding competed with a dose response of TSLP or IL-7.

Next we performed displacement binding studies using transfected 293T cells. As in the crosslinking experiments, ^{125}I-TSLP bound specifically to cells expressing both IL-7Rα and TSLPR, with excess unlabeled TSLP competing for binding of ^{125}I-TSLP. Cells transfected with the empty vector or murine *Il7r* alone showed only non-specific binding (**Fig. 5a**). Cells transfected with murine *Tslpr* alone exhibited very low binding. Analysis of binding data by Scatchard transformation was performed using the LIGAND computer program[38]. A representative experiment to determine the binding of TSLP to cells expressing TSLPR and IL-7Rα found the K_d was approximately 13 nM (**Fig. 5b**). We have performed this experiment a total of seven times and found that the K_d ranged from 1.2–40 nM. It was difficult to obtain a K_d for TSLP binding to cells expressing only the TSLPR, due to very low binding activity. We performed next a similar binding analysis using NAG 8/7 cells which constitutively express TSLPR and proliferate in response to TSLP[24,25]. As shown in **Fig. 5c**, the Scatchard transformation suggested a single class of receptors with a K_d of approximately 2.2 nM that correlates well with the data from transfected 293T cells. **Figure 5d** shows that murine IL-7 competes for binding to TSLP receptors.

Requirement of IL-7Rα for TSLP-induced proliferation

It has been suggested that IL-7Rα is involved in TSLP-induced proliferation as neutralizing antibodies against this receptor subunit inhibited proliferation of NAG8/7 cells that require the presence of TSLP or IL-7 for their growth[24]. To test directly whether expression of IL-7Rα is obligatory for TSLP-induced proliferation, we set up a reconstitution assay in 32D cells. These cells express TSLPR and γc endogenously. A stable cell line (IL-7Rα–32D) was derived from these cells by transfecting a cDNA encoding for IL-7Rα. As shown in **Fig. 6**, parental 32D cells were unable to grow in IL-7 or TSLP. However, IL-7Rα–32D cells grew in response to either TSLP or IL-7, demonstrating the absolute requirement of IL-7Rα for TSLP-induced proliferation.

TSLPR mediates signaling by TSLP

It has previously been shown that treatment of NAG8/7 cells with either IL-7 or TSLP activates *STAT5*[24,25]. To directly assess whether TSLPR was required for *STAT5* activation by TSLP, we used a functional reconstitution system in HepG2 cells. These cells were cotransfected with a CAT reporter plasmid containing a cytokine-inducible response element, *STAT5* expression vector, and either *IL7R* alone or in combination with *TSLPR* or γc. As shown in **Fig. 7**, no increase in CAT activity was seen after TSLP stimulation in the presence of IL7Rα alone or with IL-7Rα plus γc. However, if

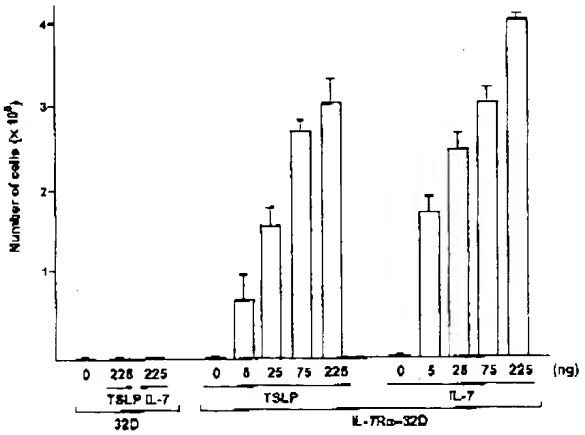

Figure 6. IL-7Rα is required for proliferation in response to TSLP. Parental 32D cells or 32D cells stable transfected with IL-7Rα (IL-7Rα–32D) were deprived of growth factor by washing in medium not containing IL-3, transferred into a 48-well dish and grown in the presence of the indicated amounts of TSLP or IL-7. The number of viable cells were counted after 7 days.

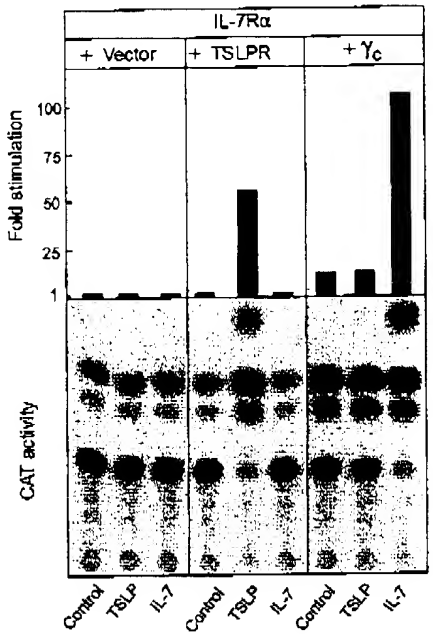

Figure 7. TSLPR is required for signaling by TSLP. HepG2 cells were transfected with expression vectors for *IL7R* and *TSLPR*, γ_c or vector alone along with plasmids for pHRRE-CAT and *STAT5B* as shown. After an overnight recovery, the cells were treated with trypsin and seeded onto six-well culture dishes. 24 h later, the cells were treated for 24 h in serum-free medium containing 100 ng/ml IL-7, or TSLP as shown. The CAT activity and fold stimulation after normalizing for transfection efficiencies are shown.

TSLPR was cotransfected, a dramatic increase in the CAT activity was observed in TSLP-stimulated cells, demonstrating that the presence of TSLPR was required for signaling. As a control, cotransfection of γ_c along with *IL7R* had no effect on TSLP-dependent reporter activity, but effectively mediated IL-7-dependent reporter activation. A result similar to that shown in **Fig. 7** was reported using a *TSLPR* cDNA cloned independently of this study[34]; however, the sequence of the *TSLPR* cDNA clone used in that study has not yet been published.

Discussion

A number of cytokine receptor chains are shared by more than one cytokine. The best known examples are gp130 (shared by IL-6, IL-11, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M and cardiotrophin-1)[35,36], β_c (shared by IL-3, IL-5 and GM-CSF)[37–39], and γ_c (shared by IL-2, IL-4, IL-7, IL-9 and IL-15)[10,17–23]. It is interesting that a total of three of the "distinctive" chains of the IL-2 family of cytokines are now known to be shared by more than one receptor. In this regard, IL-2Rβ is a component of both the IL-2 and IL-15 receptors; IL-4Rα is a component of the IL-4 and IL-13 receptors; and now IL-7Rα is established to be part of both the IL-7 and TSLP receptors. Whether this will extend to other receptor chains as well is an area for future research.

We have cloned a γ_c-like protein which cooperates with IL-7Rα to mediate signaling in response to TSLP. The TSLPR is noteworthy both for its marked similarity to γ_c and for its divergence from other type I receptors in terms of conservation of only one pair of cysteine residues and its variant WTAVT instead of WSXWS motif. In this respect, TSLPR has a distant similarity to the growth hormone receptor that contains a YGEFS instead of a WSXWS motif. The crystal structure of the growth hormone receptor complex indicated that the region containing this motif was not part of ligand-binding or receptor dimerization surfaces[40]. Saturation mutagenesis of this motif in the erythropoietin receptor showed that threonine is able to replace the two conserved serines without any effect on cell surface expression or ligand-binding of the receptor[41]. TSLP is an IL-7-like protein in that it is also secreted by stromal cells and they share certain biological properties *in vitro*. The demonstration that a functional TSLP receptor complex requires both the newly identified TSLPR and IL-7Rα suggests a possible explanation for the phenotypic differences in B cell development seen in *Il7*$^{-/-}$ and *Il7r*$^{-/-}$ mice. Both sets of mice exhibit B cell lymphopenia and a block in B cell development. However, the developmental block in *Il7r*$^{-/-}$ mice is in the pre/proB–proB transition (Hardy A–B transition), whereas in the *Il7r*$^{-/-}$ mice the block occurs later, at the C–C′ transition[42–44]. As TSLP has been shown to influence B cell development *in vitro*[24], the differences seen in these mice may be due to the actions of TSLP in *Il7r*$^{-/-}$ but not in *Il7r*$^{-/-}$ mice[22,45–47]. Determining the phenotypes of *Tslp*$^{-/-}$ or *Tslpr*$^{-/-}$ mice will assist in clarifying the exact role of TSLP. Finally, the availability of the TSLPR genetic sequence will help to elucidate the signal transduction pathways utilized by TSLP.

Methods

Cell culture, antibodies and cytokines. CTLL2 cells were grown in RPMI supplemented with 10% fetal bovine serum, antibiotics and IL-2. 293T and HepG2 cells were grown in DMEM buffer with 10% fetal bovine serum plus antibiotics. BaF3 cells were grown in RPMI supplemented with 10% fetal bovine serum, antibiotics, and 10% conditioned medium from WEHI-3 cell line as a source of IL–3. Stably transfected Kit–β cells were derived by transfecting a plasmid encoding Kit–β and selecting the cells in G418. Antibiotic-resistant clones (confirmed positive for expression of Kit–β by flow cytometry) were then infected with a retroviral vector encoding *Kit–TSLPR* and *GFP* (from an internal ribosomal entry site). These cells were then sorted for the presence of GFP positive cells. In parallel experiments, Kit–β–expressing cells were transfected with another plasmid encoding *Kit–γ* and the cells selected with hygromycin. IL-7Rα–expressing 32D cells were derived by transfecting *IL7R* cDNA into 32D cells and selecting them in puromycin. A rabbit polyclonal antiserum was generated against bacterially expressed extracellular domain of TSLPR fused in-frame with glutathione S-transferase using the pGEX4T2 expression vector (Pharmacia). IL-2 provided by Grace Ju and John Hakimi (Hoffmann La Roche, Inc., Nutley, NJ). Carrier-free IL-7 was a gift from Monica Tsang (R & D Systems, Minneapolis, MN). Recombinant TSLP was provided by D. Williams and P. Morrissey (Immunex Corp., Seattle). All other cytokines were purchased from Peprotech (Rocky Hill, NJ).

Binding and crosslinking. For affinity labeling experiments, 1 μg of TSLP was iodinated by the addition of IODO-GEN (Pierce, IL) and 2 mCi ^{125}I. The specific activity was approximately 200–300 μCi/μg. 293T cells were transiently transfected using the calcium phosphate method (5′prime–3′prime, CO). 5×10^6 cells were washed twice and 1–5 nM ^{125}I-TSLP was added. After 2 h incubation, the chemical crosslinking reagent DSS (Pierce, IL), was added at a final concentration of 0.1 mg/ml.

For displacement binding experiments, 293T cells were transfected and 1×10^6 cells were washed twice and serially diluted cold TSLP or IL-7 and a constant amount of ^{125}I-TSLP (approximately 20,000 cpm) were added. After incubating on ice for 3 h, the cells were separated from medium by centrifugation with mineral oil and *N*-butylphthalate. Cell bound cpm were measured using a gamma counter. For binding experiments using NAG8/7 cells, a similar protocol was used except that 5×10^6 cells were used. In addition, 180,000 cpm of ^{125}I-TSLP was used. Scatchard analyses were performed using the LIGAND computer program[50].

FACS analysis. The cells were grown normally and collected at 10^6/ml. They were incubated with 1:100 dilution of pre-immune or immune rabbit serum for 30 min on ice. They were then washed three times and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 min on ice. After washing three times with PBS buffer, they were analysed on a FACScan (Becton Dickinson).

Immunoprecipitations and western blotting. The cells were lysed in lysis buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, and 1 mM sodium orthovanadate in the presence of protease inhibitors. The immune complexes were captured with Protein A or G sepharose, washed in lysis buffer and resolved by SDS-PAGE. The proteins were

transferred onto nitrocellulose and the membrane blocked with 1% bovine serum albumin in PBS-buffered saline containing 0.1% Tween-20 overnight at 4 °C and then incubated with 1 μg/ml of antibody for 2 h. The membranes were incubated with secondary antibodies followed by chemiluminescent detection according to manufacturer's instructions (Amersham). In the case of NAG8/7 cells the cells were metabolically labeled with ^{35}S and lysed in 50 mM Tris buffer pH 7.4, 150 mM NaCl, 1% Triton X-100, and protease inhibitors followed by immunoprecipitation, SDS-PAGE and autoradiography. *In vitro* transcription and translation reactions were performed as described[48].

Northern blot analysis. The receptor cDNA fragment was labeled with ^{32}P and used to probe a multiple tissue northern blot (Clontech, Palo Alto and Origene Technologies, Rockville, MD) according to the manufacturer's instructions. The blot was then stripped and reprobed with a probe against beta actin.

cDNA library screening, plasmids and transfection. A murine embryo cDNA library was screened with a ^{32}P-labeled probe derived from cytoplasmic domain of *TSLPR*. One of the clones obtained contained an open reading frame that encoded *TSLPR*. This clone was sequenced completely. Additionally, several overlapping EST clones corresponding to *TSLPR* were obtained from Research Genetics and sequenced (accession numbers AA018020, AA015052, AA008678).

The extracellular and transmembrane domains of *c-Kit* and the cytoplasmic domain of *TSLPR* were amplified by polymerase chain reaction (PCR) and ligated into a retroviral vector, pMX-IRES-GFP. The constructs for Kit–β and Kit–γ have been described previously[8]. An epitope-tagged version of *TSLPR* was derived by amplifying a fragment containing the coding region by PCR using a 3' primer containing a Flag epitope. This fragment was subcloned into pCR3.1 (Invitrogen, CA) and confirmed by sequencing.

HepG2 cells were cotransfected by the calcium phosphate method with expression vectors encoding genes for IL-7Rα and TSLPR, γ_c, or vector alone along with plasmids for pHRRE-CAT containing eight tandem copies of the 27-bp cytokine-inducible hemistopoietin receptor response element and *STAT5B*[49]. After an overnight recovery, the cells were treated with trypsin and seeded onto six-well culture dishes. 24 h later, the cells were treated for 24 h in serum free medium containing 100 ng/ml IL-7, or TSLP as shown in Fig. 7. The CAT activity and fold stimulation after normalizing for transfection efficiencies is shown.

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Cloning of the Murine Thymic Stromal Lymphopoietin (TSLP) Receptor: Formation of a Functional Heteromeric Complex Requires Interleukin 7 Receptor

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Abstract

The cellular receptor for murine thymic stromal lymphopoietin (TSLP) was detected in a variety of murine, but not human myelomonocytic cell lines by radioligand binding. cDNA clones encoding the receptor were isolated from a murine T helper cell cDNA library. TSLP receptor (TSLPR) is a member of the hematopoietin receptor family. Transfection of TSLPR cDNA resulted in only low affinity binding. Cotransfection of the interleukin 7 (IL-7)Rα chain cDNA resulted in conversion to high affinity binding. TSLP did not activate cells from IL-7Rα$^{-/-}$ mice, but did activate cells from γc$^{-/-}$ mice. Thus, the functional TSLPR requires the IL-7Rα chain, but not the γc chain for signaling.

Key words: cytokine receptors • interleukin 2 receptor • interleukin 7 receptor • cytokine • DNA sequence

Introduction

The regulation of lymphopoiesis is orchestrated by a complex, overlapping network of soluble and membrane-bound molecules, among which IL-7 has been shown to play a central role (1). The effects of IL-7 are mediated by interaction with a heteromeric receptor complex consisting of a specific IL-7 binding chain (the α subunit) and the γc chain common to the IL-2, IL-4, IL-9, and IL-15 receptor complexes (2). This group of receptors, related by their common use of the γc chain, are otherwise distinct both in structure and in the range of biological activities induced by their ligands. The signaling pathways initiated by ligand binding to these receptor complexes share significant similarities, however, activating overlapping sets of kinases and other signaling molecules (3). To date, IL-4 is the only member of this group that has been demonstrated to mediate a biological signal in the absence of the γc chain, suggesting that there are both γc-dependent and -independent classes of functional IL-4 receptors (4).

As described by Sims et al. in this issue (5), we have recently identified and cloned a molecule termed thymic stromal lymphopoietin (TSLP)[1] that shares a closely overlapping, but distinct, profile of biological activities with IL-7. Given this close overlap, we initiated studies to characterize the cellular receptor for TSLP with particular attention to the possibility that these two cytokines might share common receptor components. In this paper we describe the binding characteristics of the TSLPR complex on lymphoid cells and the cloning of a TSLP-specific binding protein. We demonstrate that the IL-7Rα subunit is obligatory

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[1]*Abbreviations used in this paper:* K_a, affinity constant; RAG, recombination activating gene; SCF, stem cell factor; TSLP, thymic stromal lymphopoietin.

for formation of a high affinity, signaling competent TSLPR complex, whereas the γc chain is not required.

Materials and Methods

Cell Lines. The TSLP-dependent NAG8/7 cell line was maintained as described previously (6). BAF/BO3 cells were also maintained under these conditions, except that parental BAF/BO3 cells were supplemented with 100 ng/ml murine IL-3, and BAF/BO3 cells expressing murine IL-7 receptor were supplemented with 100 ng/ml human IL-7.

Binding Assays. Recombinant murine TSLP was expressed in mammalian cells (CV-1/EBNA) and purified as described by Sims et al. in this issue (5). Purified TSLP and murine IL-7 were radiolabeled using the enzymobead radioiodination reagent (Bio-Rad Laboratories) as described previously (7). Radiolabeled stocks were stored at 4°C in RPMI 1640 containing 2% BSA, 20 mM Hepes, and 0.2% sodium azide, pH 7.2. Binding assays were performed by a phthalate oil separation method as described previously (7). For binding assays in which TSLPR cDNA was transfected into CV-1/EBNA cells, control experiments demonstrated no difference in binding of TSLP to CV-1/EBNA cells either as adherent monolayers or in suspension; hence, all binding assays were performed with cell suspensions. CV-1/EBNA cells were transfected with receptor cDNA using DEAE-dextran followed by chloroquine treatment as described previously (8), and assayed for binding 2 d later. Nonspecific binding was assessed by including either a 1,000-fold molar excess of unlabeled TSLP and IL-7, or polyclonal rabbit anti–murine IL-7 (at a concentration previously determined to inhibit ^{125}I–IL-7 binding) into the binding mixture.

Affinity Cross-Linking. Cell suspensions were incubated with binding medium containing 0.5–1 nM ^{125}I-TSLP or ^{125}I–IL-7 for 3 h at 4°C. Nonspecific binding and cross-competition were determined by using 1,000-fold molar excess of unlabeled cytokines. After binding, cells were washed three times with PBS at 4°C, and cross-linking reagent bis-(sulfosuccinimidyl) suberate was added to a final concentration of 0.1 mg/ml; no bis-(sulfosuccinimidyl) suberate was added to control samples. Cells were cross-linked for 30–45 min at 25°C, washed once with PBS, and extracted in PBS/1% Triton containing 1 mM PMSF, 10 μM pepstatin A, 10 μM leupeptin, 2 mM *O*-phenanthroline, 1 mM EGTA, 0.5 mM EDTA, and 0.02% sodium azide for 20–30 min at 4°C. Lysed cells were centrifuged at 12,000 *g* for 15 min at 4°C, and supernatants were retained. Supernatants were then analyzed by autoradiography after 8–16% gradient SDS–PAGE as described previously (9).

BAF/BO3 Transfection and Proliferation Assays. BAF/BO3 cells were transfected with murine IL-7Rα cDNA by electroporation as described (10). In brief, 8×10^6 cells were washed in RPMI 1640, 10 μg murine IL-7Rα cDNA was added (11), electroporated at 280 V and 960 μF using a Bio-Rad Laboratories gene pulser, and selected in 100 ng/ml human IL-7. For proliferation assays, parental and transfected BAF/BO3 cells were cultured in 96-well flat-bottomed plates (1×10^4 cells/well) with serial threefold dilutions of murine IL-3, TSLP, or human IL-7. The starting concentration of cytokines was 10 μg/ml; background was measured by adding cells to assay medium alone. Cells were cultured for 2 d, pulsed for 5 h with ^{3}H-TdR, harvested onto glass fiber filters, and incorporation was determined.

cDNA Cloning. An oligo-dT–primed cDNA library from the murine T helper cell clone 7B9 (12) was transfected into CV1/EBNA cells and screened with ^{125}I-TSLP essentially as described (13). A chloroquine-mediated DEAE-dextran method (8) was used to transfect subconfluent monolayers of CV-1/EBNA cells grown in pronectin-treated chamber slides, with miniprep DNA from pools of 1,000 clones from the 7B9 library. Since the 7B9 library was in an expression vector that contained the SV40 origin of replication but lacked the Epstein-Barr virus origin, the library plasmids (2.5 μg per slide) were cotransfected with pSV3neo (14) (0.5 μg) as a source of T antigen to allow the plasmid to replicate and thereby increase both the transfection efficiency and the expression level (15). After 2 d of expression, slides of transfected cells were incubated with 1 nM ^{125}I-TSLP in binding medium containing 5% nonfat dry milk for 1 h at 25°C. The slides were then washed with PBS, fixed with 2.5% glutaraldehyde in PBS, washed, air-dried overnight, and dipped in liquid photographic emulsion. After 3 d of exposure in the dark at 25°C, the slides were developed and examined at ×10–20 magnification for the presence of silver grains over cells that have bound to ^{125}I-TSLP. Positive pools were broken down and single clones were isolated as described (13). TSLPR sequence data are available from EMBL/GenBank/DDBJ under accession no. AF232936.

RNA Hybridization. PolyA$^+$ RNA (5 μg) was run on agarose gels, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled antisense riboprobe derived from the entire insert of clone 7a as described (13). The final wash of the filters was for 60 min at 63°C in 0.1× SSC.

Interspecific Mouse Backcross Mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (16). A total of 205 N_2 mice were used to map the *Tslpr* locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (17). All blots were prepared with Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech). The probe, a 1.35-kb EcoRI/HindIII fragment of mouse cDNA, was labeled with [α-^{32}P]dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of 0.25× SSCP, 0.1% SDS, 65°C. A major fragment of 3.3 kb was detected in PvuII-digested C57BL/6J DNA and a major fragment of 3.7 kb was detected in PvuII-digested *M. spretus* DNA. The presence or absence of the 3.7-kb PvuII *M. spretus*–specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Tslpr* including *Bmp3*, *Gfi 1*, and *Adrbk2* has been reported previously (18, 19). Recombination distances were calculated using Map Manager, v2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Whitlock-Witte Culture Analysis. Livers were isolated from newborn IL-7Rα$^{-/-}$ and wild-type C57BL/6 mice. The derivation and description of mice with a targeted disruption of the IL-7Rα gene have been described (20). Cultures were set up with a modification of the method of Whitlock and Witte (21). In brief, livers were disrupted into a single cell suspension and cultured in RPMI 1640 plus 5% charcoal filtered FCS (Hyclone) containing human IL-7 (10 ng/ml), or murine stem cell factor (SCF) (1 μg/ml), or murine TSLP (100 ng/ml), or SCF plus TSLP in 60-mm tissue culture dishes (Costar) and incubated at 37°C for 9 d. Phenotypic analysis was performed with FITC-conjugated RA3-6B2, GR-1, and Mac-1 Abs. Samples were analyzed on a FACScan™ (Becton Dickinson) using either a lymphoid- or myeloid-specific gate as determined by forward versus side light scatter profiles. The lymphoid gate was defined by the light scatter of the cells

that grew out under IL-7 stimulation, and the myeloid gate was defined by the light scatter of the cells that grew out under SCF stimulation.

Thymocyte and Bone Marrow Proliferation Assays. Unfractionated thymocytes from mice deficient in γc (22) or wild-type controls were cultured in 96-well plates (2×10^5 cells/well). Cells were stimulated with ConA (5 μg/ml) alone or in combination with IL-7 (10 ng/ml), or TSLP (10 and 0.4 ng/ml). Cells were cultured for 72 h and were pulsed with ^{3}H-TdR for the last 6 h. Mice deficient in both γc and recombination activating gene (RAG)-2 (23) were obtained through intercrossing, and mutants were identified by PCR using genomic DNA derived from tail snips. Bone marrow was flushed from femurs and tibias and the cells were stained with PE-conjugated anti-B220 Ab. B220$^+$ B cell lymphoid precursors were isolated by cell sorting using a FACStar^{PLUS}™ (Becton Dickinson). B cell cultures were initiated by plating 10^6 sorted B220$^+$ cells on mitomycin C-treated ST2 stromal cells (24) supplemented with TSLP (100 ng/ml). After a period of 10 d, growth of clusters of round lymphoid cells was observed which were found to consist of >95% B220$^+$ lymphocytes. Nonadherent cells (5×10^4) were washed and replated in 96-well round-bottomed plates in 200 μl of RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, nonessential amino acids, and antibiotics, and the following growth factor combinations: muIL-7 (50 ng/ml), TSLP (100 ng/ml), and SCF (100 ng/ml). Cells were cultured for 2 d and pulsed with ^{3}H-TdR for the last 12 h.

Results

Characterization of TSLP Receptors on Lymphoid Cell Lines. Purified recombinant murine TSLP was iodinated and shown to exhibit specific binding to both B and T lymphoid cell lines. Typical equilibrium binding data for ^{125}I-TSLP at 37°C to the murine pre-B cell line 70Z/3 and the murine T cell line 7B9 is shown in Fig. 1, A and B. Conditions chosen for binding reflected preliminary experiments that showed that binding was saturable and required <60 min to reach equilibrium at either 37 or 4°C. In both cases, Scatchard analysis of the data indicated a single class of high affinity binding sites for TSLP (25). For 70Z/3 cells, the calculated affinity constant (K_a) was $7.1 \pm 1.2 \times 10^9$ M^{-1} with 1,200 ± 900 sites per cell (average of 12 experiments), and for 7B9 cells, the K_a was $1.1 \pm 0.1 \times 10^{10}$ M^{-1} with 1,200 ± 700 sites per cell (average of 2 experiments). Binding experiments with 70Z/3 cells were also performed at 4°C and produced binding constants very similar to those obtained at 37°C (data not shown). These results are in contrast to those previously obtained with murine IL-7 where binding to the pre-B cell line IxN/2b produced complex biphasic curves at either 4 or 37°C, indicative of both high and low affinity IL-7 binding sites on these cells (9).

TSLPR expression was evaluated on a variety of murine and human cell lines by radioligand binding (Table I). TSLP binding by murine cell lines of both lymphoid and myelomonocytic origin was detected at relatively low levels. Most cell lines that bound ^{125}I-TSLP exhibited a single high affinity binding site with a K_a between 0.4 and 1×10^{10} M^{-1}. Interestingly, in some murine cell lines (LSTRA, NS1.1, P815), a low affinity binding component was also detected. This affinity was difficult to measure accurately

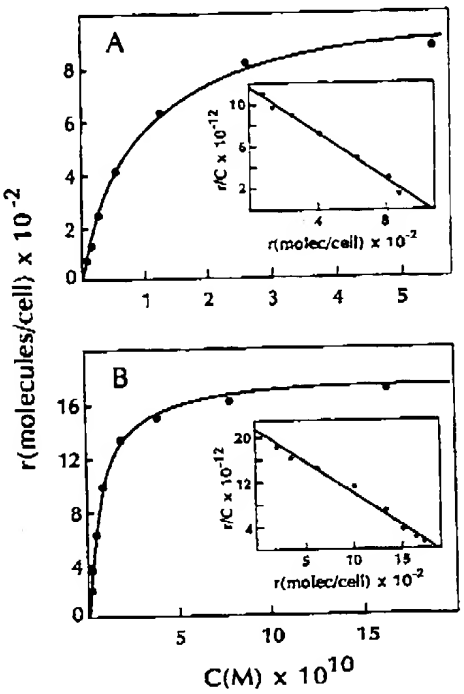

Figure 1. Equilibrium binding of ^{125}I-TSLP to lymphoid cell lines. 70Z/3 cells (A) and anti-CD3–stimulated 7B9 cells (B) were incubated with various concentrations of ^{125}I-TSLP for 30 min at 37°C. Binding was assayed as described in Materials and Methods. All data were corrected for nonspecific binding. Insets show Scatchard representations of specific binding from each panel.

and was $<4 \times 10^8$ M^{-1}. These results suggest that the TSLPR complex may consist of a low affinity binding subunit that is converted to high affinity when complexed with an additional receptor chain, as has been previously shown for many other cytokine receptors (2). Interestingly, cell lines that bound TSLP were also known to bind murine IL-7 (9). However, unlike murine IL-7, which is capable of binding to IL-7 receptors on human cells, none of the human lines tested was found to bind murine TSLP. Given that these lines represented a panel of both lymphoid and myelomonocytic cell types quite analogous to the murine lines that bind TSLP, these data would suggest quite strongly that murine TSLP is incapable of cross-reacting with a putative human TSLPR.

To explore the possibility that TSLP might interact with subunits of the IL-7 receptor, inhibition studies were performed in which unlabeled TSLP, murine IL-7, and human IL-7 were tested for their ability to inhibit binding of ^{125}I-TSLP to 70Z/3 cells. Both murine and human IL-7 were capable of inhibiting ^{125}I-TSLP binding, although not as effectively as unlabeled TSLP (Fig. 2), which exhibited an inhibition constant of 9.8×10^9 M^{-1}. Also, murine IL-7 was more effective than human IL-7 at competing for TSLP binding. These data suggest that TSLP was able to interact with at least one component of the IL-7 receptor complex. To gain fur-

Table I. *Distribution of TSLPRs*

Cell line	Characteristic	TSLP bound (molecules/cell) High K_a*	TSLP bound (molecules/cell) Low K_a*
Murine cells			
Nag8/7	TSLP-dependent pre-B cell	500–1,700	nd‡
70Z3	Pre-B cell	300–3,000	nd
IxN/2B	IL-7–dependent pre-B cell	700–1,500	nd
NFS-25-C3	Pre-B lymphoblast	440	nd
NFS-5-C1	Pre-B lymphoblast	480	nd
BAF/BO3	Pre-B cell	nd	250–2,500
Wehi 279	B cell	nd	nd
7B9	T cell clone (helper)	500–1,800	nd
CTLL	IL-2–dependent T cell	nd	nd
EL-4	T cell thymoma	nd	nd
LSTRA	T lymphocytic leukemia	35	7,500
FDCP-2	Myeloid	nd	nd
FDCP-1	Promyeloid	nd	210
PU5-1.8	Macrophage	1,000	nd
P388D1	Macrophage	nd	nd
J774	Macrophage	nd	300
Raw 264.7	Monocytic	nd	nd
NS1.1	Monocytic	100	1,400
P815	Mastocytoma	20	1,300
MC-6	Mast cell	nd	540–2900
DA-1	Mast cell	nd	nd
Human cells			
JM-1	Pre-B cell	nd	nd
EU.1	Pre-B cell	nd	nd
Daudi	B cell lymphoma	nd	nd
Raji	B cell lymphoma	nd	nd
CB23	B cell lymphoma	nd	nd
MP-1	B cell lymphoma	nd	nd
THP-1	Monocytic	nd	nd
U937	Monocytic	nd	nd
MO7E	Premegakaryocytic	nd	nd
TF-1	Proerythrocytic	nd	nd
KG-1	Myelogenous leukemia	nd	nd
W126	Lung fibroblast	nd	nd
IMTLH	Stromal	nd	nd

Binding experiments were conducted as described in Materials and Methods. Molecules bound per cell were determined by Scatchard analysis of complete sets of binding data and represent total sites per cell.
*High and low affinity sites are those defined as having K_a values $>4 \times 10^9$ and $<4 \times 10^8$ M^{-1}, respectively.
‡nd, not detected. Limit of detection is defined as 20 molecules bound per cell.

ther understanding of the nature of the TSLPR, we undertook to clone the cDNA for a specific TSLP binding chain.

Isolation of a cDNA Clone Encoding a TSLP Binding Chain. Cloning of a cDNA encoding the TSLPR was accomplished by direct expression in mammalian cells (26). A cDNA library was generated in an expression vector using mRNA from a murine T helper cell clone (7B9) that binds radiolabeled TSLP. Pools of cDNA clones were transfected into CV-1/EBNA cells and 3 d later the cells were incubated with ^{125}I-TSLP, with the expectation that if a pool contained a functional TSLPR cDNA, expression of that clone would result in TSLP binding to some of the transfected CV-1/

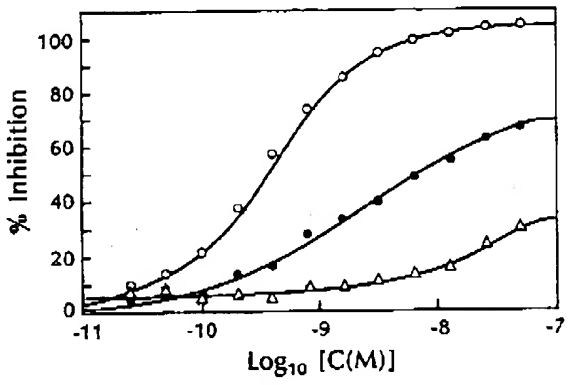

Figure 2. Inhibition of ^{125}I-TSLP binding by unlabeled TSLP and IL-7. 70Z/3 cells (5×10^7 cells) were incubated with ^{125}I-TSLP (2.6×10^{-13} M) and varying concentrations of unlabeled TSLP (○), murine IL-7 (●), and human IL-7 (△) for 30 min at 37°C. Binding was assayed as described in Materials and Methods. The continuous curves were calculated from either a one-site (TSLP) or two-site (murine and human IL-7) competitive inhibition equation using a K_a value for ^{125}I-TSLP of 8.8×10^9 M^{-1}. All data were corrected for nonspecific binding.

EBNA cells. Two positive pools were found after screening 50,000 cDNA clones, which were then broken down into successively smaller subpools until a single cDNA clone encoding a TSLPR was isolated from each (clones 7a and 19). The inserts from the two clones were sequenced and found to be identical in the extensive region of overlap.

The longer of the two TSLPR clones, clone 7a, had a single open reading frame of 359 amino acids (Fig. 3 A). The sequence predicts a type I transmembrane protein with a hydrophobic signal peptide at the NH_2 terminus and a single membrane-spanning segment. The extracellular portion is a member of the hematopoietin receptor family (27) that includes the receptors for many other cytokines. The TSLPR lacks the second of the four conserved cysteines present in other hematopoietin family members, and in place of the hallmark WSXWS motif contains the sequence WTAVT. There are two potential sites for N-linked glycosylation. The cytoplasmic portion is predicted to be 106 amino acids in length. It contains the "box 1" region (28) that is common to all hematopoietin family receptors and is thought to be a binding site for signal transducing molecules. In addition, the TSLPR shows a more extended homology with the cytoplasmic regions of the erythropoietin receptor, the IL-9 receptor, and the β chain of the IL-2 receptor (Fig. 3 B). There is a formal possibility that the natural TSLPR protein is larger than shown in Fig. 3 A, since no in-frame stop codons are present in the 5′ untranslated region of clone 7a. If that is the case, however, the additional NH_2-terminal amino acids would seem to be unnecessary for proper cell surface membrane expression and TSLP binding activity of the molecule.

Expression of TSLPR mRNA in Murine Tissues and Cell Lines. Northern blots reveal a single hybridizing RNA species that migrates somewhat faster than 18S rRNA (Fig. 4), consistent with the mRNA size of ~1,300 nucleotides

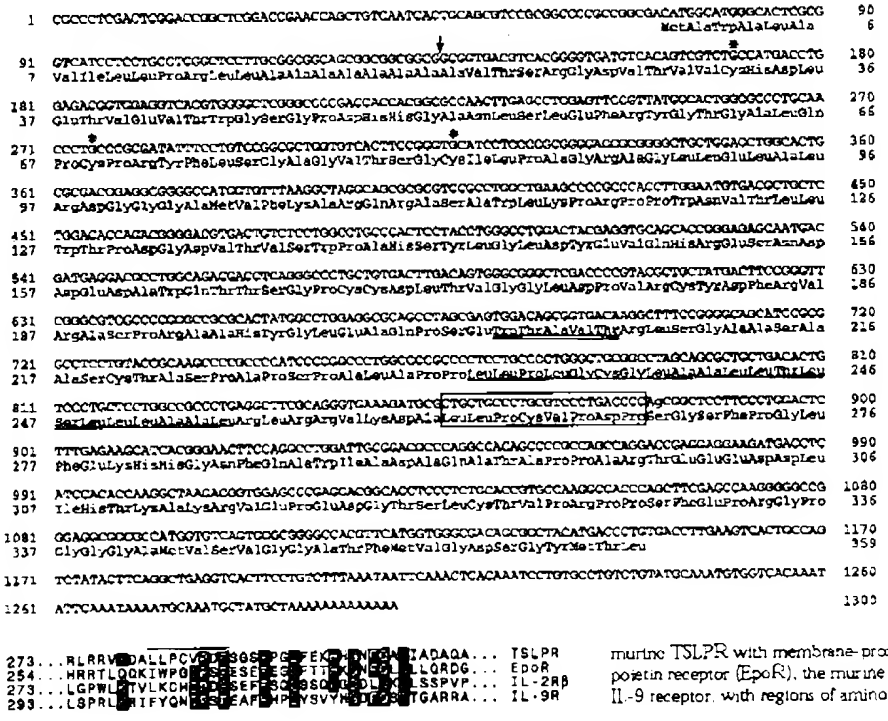

Figure 3. Murine TSLPR cDNA. (A) The cDNA and predicted amino acid sequence of the murine TSLPR clone 7a. Signal peptide cleavage is shown by the arrow as occurring after Ala 21, although cleavage after Ala 15 would be nearly as preferred (reference 37). The transmembrane region is underlined; asterisks mark the cysteines conserved in hematopoietin family members; a double underline indicates the sequence WTAVT, present here in place of the more typical WSXWS; the box 1 sequence of Murakami et al. is boxed (reference 28). A second functional cDNA clone isolated in the expression screen, clone 19, extends from nucleotide 80 through the polyA stretch at the 3′ end. The initiating Met-Ala-Trp sequence at the NH_2 terminus of clone 7a is replaced in clone 19 by Met-Gly, encoded in the adapter used to insert the cDNA into the vector. (B) Alignment of the membrane-proximal segment of the murine TSLPR with membrane-proximal segments of the murine erythropoietin receptor (EpoR), the murine IL-2 receptor β chain, and the murine IL-9 receptor, with regions of amino acid conservation highlighted.

(excluding polyA) estimated from the cDNA clones. TSLPR mRNA was detected in EL/4 and 70Z/3 cells, thymus, spleen, and weakly in kidney and bone marrow. It was not detected in 3T3 cells, LMtk$^-$ cells, brain, and liver (Fig. 4). Expressed sequence tags derived from TSLPR mRNA have been found in mouse cDNA libraries made from placenta, embryo, embryonic stem cells, testes, small intestine, brain, and macrophages.

Chromosomal Location of Tslpr. The mouse chromosomal location of *Tslpr* was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6J × *M. spretus*, F_1 × C57BL/6J) mice (16). The mapping results indicated that *Tslpr* is located in the central region of mouse chromosome 5 linked to *Bmp3*, *Gfi 1*, and *Adrbk2* (data not shown). The most likely gene order is: centromere - *Bmp3* - *Gfi 1* - *Tslpr* - *Adrbk2*.

Expression and Characterization of TSLPR cDNA. Evidence that the isolated TSLPR clone 7a encoded a functional TSLP binding protein is shown in Fig. 5, A and B. TSLPR clone 7a expressed alone in transfected CV-1/EBNA cells was found to bind ^{125}I-TSLP with low affinity only, displaying a single low affinity site with a K_a of 1.3 ± 1.2 × 10^8 M^{-1} (average of six experiments). This affinity is similar to that observed for ^{125}I-TSLP binding to a limited subset of murine cell lines such as BAF/BO3 and MC6. To determine if the IL-7Rα subunit might be a component of the TSLPR complex that would increase the affinity of TSLP binding, TSLPR cDNA was cotransfected into CV-1/EBNA cells with either murine (Fig. 5 A) or human (Fig. 5 B) IL-7Rα cDNA clones. Binding analysis with ^{125}I-TSLP revealed that cells coexpressing the TSLPR and either murine or human IL-7Rα proteins exhibited biphasic binding characteristics with both high and low affinity binding sites. The new high affinity site had a K_a of 1.8 ± 1.1 × 10^{10} M^{-1} (average of five experiments), which is similar to that observed for ^{125}I-TSLP binding to lymphoid and myelomonocytic cells, suggesting that the native high affinity TSLPR complex might be comprised of just these two components.

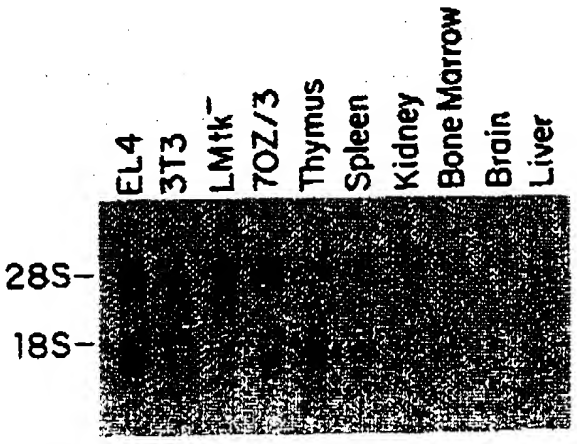

Figure 4. Northern blot analysis of TSLPR mRNA. PolyA$^+$ RNA from various cell lines and tissues was hybridized to an antisense TSLPR riboprobe as described in Materials and Methods. The band at 28S reflects the cross-hybridization of the TSLPR probe to residual rRNA present in the samples.

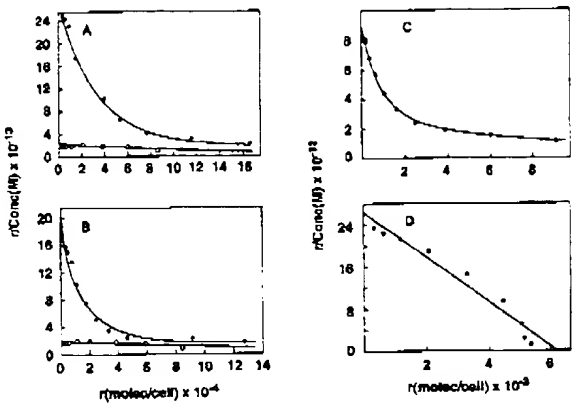

Figure 5. Binding characteristics of TSLPR with the IL-7Rα chain. (A and B) Binding of ^{125}I-muTSLP to CV-1/EBNA cells transfected with murine TSLPR with (●) or without (○) murine IL-7Rα (A) or human IL-7Rα (B). (C and D) Binding of ^{125}I-muIL-7 (C) or ^{125}I-muTSLP (D) to BAF/BO3 cells transfected with the murine IL-7Rα. In all cases binding was done for 30 min at 37°C and assayed as described in Materials and Methods.

This hypothesis was further explored by taking advantage of the observation that BAF/B03 cells demonstrate only low affinity binding for ^{125}I-TSLP (K_a < 4 × 10^8 M^{-1}) and lack the IL-7Rα chain as they do not bind ^{125}I-IL-7 (data not shown). Additionally, this indicates that IL-7 does not bind to TSLPR. These cells were stably transfected with murine IL-7Rα cDNA as described above (see Materials and Methods), and the binding affinities for ^{125}I-muIL-7 and ^{125}I-TSLP were measured (Fig. 5, C and D). ^{125}I-IL-7 binding was very similar to that described previously for other murine cell lines, exhibiting a biphasic Scatchard plot (9). The curve shown in Fig. 5 C had 1,100 high affinity sites with a K_a of 7 × 10^9 M^{-1}, and a large number of low affinity sites (>20,000) with an affinity of <1 × 10^8. In contrast, binding of ^{125}I-TSLP to these transfected cells (Fig. 5 D) showed a single high affinity site with a K_a of 3.9 ± 0.4 × 10^9 M^{-1} with 4,500 ± 3,100 sites/cell (average of four experiments). This affinity is in the same range as that measured for TSLP binding on the various lymphoid and myelomonocytic cell lines shown in Table I. In addition, it is apparent from the ^{125}I-IL-7 binding curve that the IL-7Rα chain is expressed in apparent excess to the number of endogenous TSLPR chains. This ratio would explain why all the low affinity TSLPR chains are effectively converted to high affinity TSLP binding complexes by the presence of the IL-7Rα chain, and suggests that in most of the cell lines we tested for TSLP binding, the IL-7Rα is also expressed in excess relative to the TSLPR chain.

Characterization of the TSLP Receptor by Affinity Cross-Linking. To further characterize the TSLPR complex, we performed affinity cross-linking studies on a cell line (70Z/3) containing endogenous high affinity TSLPRs, on CV-1/EBNA cells that had been transfected with only the

TSLPR clone 7a, and on BAF/BO3 cells (which express endogenous TSLPR chain) transfected with the murine IL-7R cDNA. On 70Z/3 cells (Fig. 6 A), cross-linking with ^{125}I-TSLP revealed two major species with molecular masses of ~100 and 75 kD. Cross-linking of ^{125}I-TSLP in both of these bands is relatively evenly reduced in the presence of unlabeled murine or human IL-7 (Fig. 6 A, lanes c and d). Incubation in the presence of unlabeled TSLP totally eliminates the presence of both cross-linked species (data not shown). After subtraction of the molecular mass of TSLP (major species of 23 kD, with minor species of 18 kD due to differential glycosylation), the cross-linked species would correspond to membrane proteins of ~75 and 50 kD, respectively. The larger species corresponds closely to the size of the murine IL-7R chain as previously measured by affinity cross-linking, suggesting that the smaller species may represent the TSLPR chain (9, 11). This hypothesis is substantiated by the results obtained from cross-linking TSLP to CV-1/EBNA cells transfected with the TSLPR clone 7a alone (Fig. 6 B). A single cross-linked species is observed that corresponds in size to the smaller of the two species seen on the 70Z/3 cells (Fig. 6 B, lane f), and cross-linking of ^{125}I-TSLP to this species is completely inhibited by the inclusion of unlabeled TSLP in the incubation mixture (Fig. 6 B, lane g). Cross-linking to BAF/BO3 cells that contain endogenous TSLPR and transfected recombinant murine IL-7Rα produced a pattern of cross-linking with ^{125}I-TSLP essentially identical to that seen on the 70Z/3 cells (Fig. 6 C, lane i). Cross-linked complexes of ~100 and 75 kD are observed, which are competitively inhibited by unlabeled TSLP (Fig. 6 C, lane j). To elucidate the nature of the higher molecular mass cross-linked species, these cells were also cross-linked with ^{125}I-muIL-7. As shown in Fig. 6 C, lane k, two main cross-linked species were observed, one of ~100 kD and a larger species of ~180 kD. This pattern is very similar to what was previously observed for ^{125}I-IL-7 cross-linking (9), where we proposed that the higher molecular mass species may correspond to cross-linking of ^{125}I-IL-7 to a dimer of IL-7Rα chains. The 100-kD cross-linked species corresponds closely in size to the larger of the two species cross-linked with ^{125}I-TSLP. Taken together, these data provide strong evidence that the expressed natural and recombinant TSLPR chains have a molecular mass of ~50 kD, and that in cells expressing both the TSLPR and IL-7Rα chains, TSLP is capable of interacting with both.

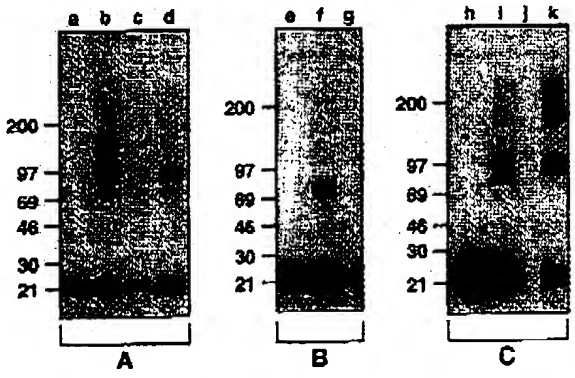

Figure 6. Characterization of the TSLPR by affinity cross-linking. (A) 70Z/3 cells were incubated with ^{125}I-TSLP in the absence of unlabeled competitor (a and b), or in the presence of unlabeled murine IL-7 (c) or human IL-7 (d) and then treated with (b–d) or without (a) cross-linker. (B) CV-1 cells transfected with TSLPR clone 7a were incubated with ^{125}I-TSLP in the absence of unlabeled competitor (e and f) or in the presence of unlabeled TSLP (g) and then treated with (f and g) or without (e) cross-linker. (C) BAF/BO3 cells transfected with muIL-7Rα were incubated with ^{125}I-TSLP in the absence of unlabeled competitor (h and i) or in the presence of unlabeled TSLP (j), or with ^{125}I-IL-7 (k) and then treated with (i–k) or without (h) cross-linker. Cross-linking was performed as described in Materials and Methods and samples were run on SDS-PAGE under reducing conditions using 8–16% gradient gels and analyzed by autoradiography.

Signal Transduction Mediated by TSLPR Complexes. Having shown that the TSLPR is a low affinity TSLP binding chain that can be converted to a high affinity binding complex in the presence of the IL-7Rα chain, we next wanted to examine the signaling capabilities of these two forms of the TSLPR. BAF/BO3 cells (which express TSLPR and γc) that were transfected with the IL7Rα were tested for their response to IL-3, IL-7, and TSLP. Parental BAF/BO3 cells that proliferated in response to IL-3 were incapable of responding to either human IL-7 or TSLP even when these factors were used at very high concentrations (data not shown). BAF/BO3 cells transfected with the IL-7Rα now proliferated to both IL-7 and TSLP (Fig. 7). Although these cells proliferated to a greater extent to IL-7, their response to TSLP was significantly greater than background (cells with no added growth factor, $P < 0.05$) at all concentrations of TSLP >1.5 ng/ml. It therefore appears, at least in this cell population, that the IL-7Rα chain is required for generation of a functional TSLPR complex.

Given that the functional IL-7Rα complex has also been shown to contain the γc chain (29), it was necessary to determine if the functional TSLPR complex requires the γc chain and thus exists as a heterotrimer with TSLPR and IL-7Rα, or whether the TSLPR and IL-7Rα chains are sufficient for signaling. BAF/BO3 cells endogenously express the γc chain, so the proliferative results in the IL-7Rα–transfected BAF/BO3 line were not informative. To determine the requirement for the IL-7Rα and γc chains in formation of a functional TSLPR, we therefore examined the ability of TSLP to mediate biological activities on cells from mice that were deficient in either the IL-7Rα chain or the γc chain.

Analysis of TSLP Activity in Whitlock-Witte Cultures Derived from IL-7Rα–deficient Mice. Whitlock-Witte cultures were established using murine neonatal liver as the source of hematopoietic cells from wild-type or IL-7Rα$^{-/-}$ mice (30, 31). These cells were cultured with TSLP, IL-7, SCF, or TSLP plus SCF for 9 d. Nonadherent cells were then harvested, counted, and analyzed by immunoflourescent staining and flow cytometry.

As can be seen in Fig. 8 A, compared with IL-7 or SCF,

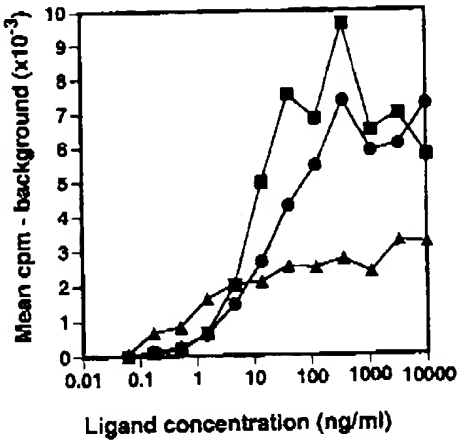

Figure 7. Proliferative response of BAF/BO3 cells transfected with the muIL7Rα chain. BAF/BO3 cells expressing murine IL-7Rα chain (2×10^5 cells/ml) were cultured in medium alone, murine IL-3 (■), human IL-7(●), or murine TSLP (▲) for 2 d. Cells were then pulsed for 5 h with ^{3}H-TdR and harvested. The background cpm (cells in medium alone) averaged 184 cpm.

TSLP did not stimulate significant growth of either wild-type or IL-7Rα–deficient cells in this culture system. However, the nonadherent cell yield in response to TSLP by both wild-type and IL-7Rα–deficient cells is greater than in cultures with no added cytokine in which only an adherent cell layer develops. The combination of TSLP and SCF did not result in a greater degree of nonadherent cell growth than with SCF alone in either wild-type or IL-7Rα–deficient cells. Interestingly, phenotypic analysis of these cultures showed that cells grown in SCF or SCF plus TSLP had a roughly similar distribution of myeloid cells (Fig. 8 B, myeloid gate, Mac-1 and Gr-1 staining). However, wild-type cells grown in SCF plus TSLP had a significant population of B220$^+$ cells (Fig. 8 B, lymphoid gate) that was not present in cultures grown in SCF alone (an insignificant number of events was present in the lymphoid gate to accumulate data; Fig. 8 B). IL-7Rα$^{-/-}$ cells cultured with SCF and TSLP resulted in only myeloid cell growth (an insignificant number of events was present in the lymphoid gate to accumulate data; Fig. 8 B). Thus, these data demonstrate that TSLP alone does not stimulate significant growth in Whitlock-Witte culture conditions, but in the presence of SCF results in significant lymphoid growth, compared with SCF alone which stimulates only myeloid growth. This lymphoid population is not observed in cultures established with IL-7R$^{-/-}$ cells, further supporting the hypothesis that TSLP-mediated signaling requires the IL-7Rα chain.

Analysis of TSLP Activity on Cells from γc-deficient Mice. To determine if the γc chain was involved in the TSLPR signaling complex, the proliferative response of wild-type and γc$^{-/-}$ lymphocytes in response to TSLP was assessed. It has been previously demonstrated that γc$^{-/-}$ thymocytes proliferate poorly in response to ConA, and that this response cannot be augmented by the addition of exogenous IL-2, IL-4, or IL-7 (22). As can be seen in Table II, the addition of TSLP to ConA resulted in a significant increase in proliferation of γc$^{-/-}$ thymocytes, suggesting that γc is not required for TSLP-mediated signaling. This observation was confirmed using B cell precursors from RAG-2–deficient mice (23). These mice accumulate B cell precursors due to an inability to rearrange Ig genes precisely at the point where they are most responsive to the combination of IL-7 and SCF. Thus, long term cultures of RAG-2–deficient B cell progenitors can be maintained in vitro with IL-7 and SCF (32). B220$^+$ bone marrow cells from RAG-2$^{-/-}$ or γc$^{-/-}$RAG-2$^{-/-}$ double mutants were cultured on ST2 stromal cells supplemented with TSLP. The nonadherent lymphoid cells that expanded expressed B220 and IL-7Rα and were negative for surface IgM, Gr-1, and Mac-1 (data not shown). As can be seen in Table II, these enriched B220$^+$ B cell precursors from γc$^{-/-}$RAG-2$^{-/-}$

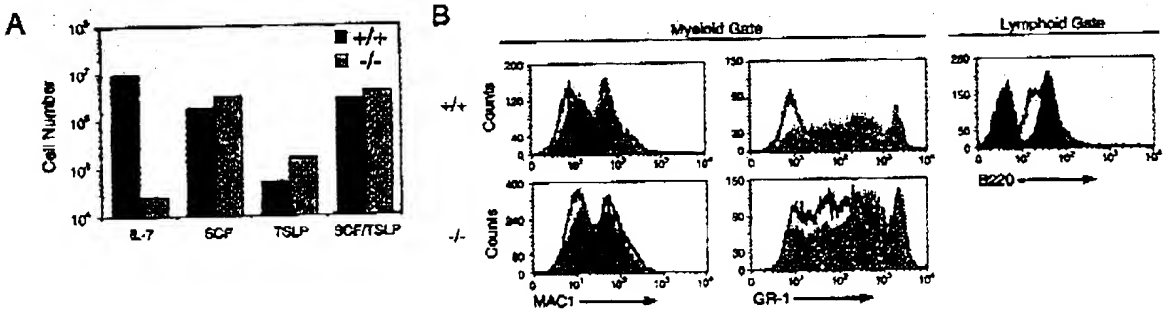

Figure 8. Analysis of TSLP activity in Whitlock-Witte cultures derived from wild-type or IL-7Rα$^{-/-}$ mice. (A) Livers from IL-7Rα$^{-/-}$ or wild-type newborn mice were grown under Whitlock-Witte culture conditions for 9 d in the presence of either IL-7 (10 ng/ml), SCF (1 μg/ml), TSLP (100 ng/ml), or SCF plus TSLP. Enumeration of nonadherent cells was determined by trypan blue exclusion. (B) Immunofluorescent profiles of nonadherent cells isolated from IL-7Rα$^{-/-}$ and wild-type mice. Lymphoid and myeloid gates were established by the forward versus side light scatter profiles of cells grown in IL-7 alone or SCF alone, respectively. Myeloid gated cells were analyzed for Mac-1 and Gr-1 expression after growth in SCF (gray histogram) or SCF plus TSLP (white histogram). Lymphoid gated cells were analyzed for B220 expression after growth in IL-7 (hatched histogram) or SCF plus TSLP (white histogram). Black histogram represents IgG_1 isotype control staining of cells grown in IL-7.

Table II. *Proliferative Response of Thymocytes and B220⁺ Cells from γc⁻/⁻ Mice to TSLP*

	$\gamma c^{-/-}$	$\gamma c^{+/+}$
	cpm × 10^{-3}	
Thymocytes		
ConA	4.7	7.2
ConA + IL-7	4.6	25.6
ConA + TSLP 10 ng/ml	11.5	15.3
ConA + TSLP 0.4 ng/ml	11.2	10.9
PMA/Ionomycin	49.5	52.4
PMA/IL-4	0.2	44.1
B220⁺ cells (RAG-2⁻/⁻)		
SCF	0.2	0.5
IL-7	0.4	11.2
IL-7/SCF	0.5	20.3
TSLP	1.7	1.6
TSLP/SCF	11.6	5.6

Enriched B220⁺ cells are from $\gamma c^{-/-}$RAG-$2^{-/-}$ and $\gamma c^{+/+}$RAG-$2^{-/-}$ mice (see Materials and Methods). The values are the arithmetic mean of triplicate wells and are from a single representative experiment.

mice were able to proliferate in response to TSLP in the presence of SCF, but not to IL-7 in the presence of SCF. These data demonstrate that the γc is not required for the response to TSLP by thymocytes and B cell precursors under these culture conditions.

Discussion

TSLP is a cytokine that shares a closely overlapping, but distinct, profile of biological activities with IL-7 (6). In Sims et al. in this issue, we have reported the cloning and further characterization of this molecule (5). In this report, we utilize purified recombinant-derived murine TSLP to characterize the cell surface receptor complex to which this cytokine binds, and report cloning of a cDNA encoding a specific murine TSLP binding chain. Equilibrium binding studies with radiolabeled TSLP demonstrated that a variety of murine lymphoid, myeloid, and monocytic cell lines were capable of binding TSLP, in most cases exhibiting a single high affinity binding site. However, there were a few cell lines that exhibited both a high and low affinity site, and three lines that exhibited low affinity binding only. This pattern of binding is reminiscent of several other cytokine receptors that contain two or more subunits, in particular those that contain a specific low affinity binding chain, and an additional converting subunit that generates a high affinity, signal tranduction competent complex.

Given the apparent biological relationship of TSLP to IL-7, it was also interesting to note that all the cell lines that were found to bind TSLP had been previously demonstrated to bind IL-7. This suggested the possibility that the receptors for TSLP and IL-7 might share one or more common subunits. Subsequent binding inhibition and cross-linking studies substantiated this hypothesis and led to our efforts to clone a receptor subunit that might specifically bind TSLP. A cDNA clone encoding a TSLP binding chain (TSLPR) was isolated by direct expression from a murine 7B9 T helper cell clone cDNA library. The sequence predicts a type I transmembrane protein that is clearly a member of the hematopoietin receptor family, but with interesting variations. The TSLPR lacks the second of the four conserved cysteines present in other hematopoietin family members, and in place of the hallmark WSXWS motif it contains the sequence WTAVT. The cytoplasmic portion contains the "box 1" region that is common to all hematopoietin family receptors and is thought to be a binding site for signal transducing molecules (28). In addition, the TSLPR sequence shows extended homology with the cytoplasmic regions of the erythropoeitin receptor, the IL-9R, and the β chain of the IL-2R.

Following up on the possibility that TSLP and IL-7 might share common receptor subunits, the binding of TSLP to TSLPR, both in the presence and absence of IL-7Rα, was examined by expressing these subunits in CV-1/EBNA cells. The results showed that the TSLPR subunit alone bound TSLP with low affinity whereas the combination of TSLPR and IL-7Rα generated a high affinity site. The affinities of both these sites matched those previously measured in murine cell lines. Interestingly, both the murine and human IL-7Rα subunits were equally effective in combining with the murine TSLPR subunit to generate a high affinity murine TSLP binding complex. This was somewhat surprising since extensive binding studies failed to identify any human cell lines capable of binding murine TSLP, indicating that this molecule exhibits strict species specificity. In contrast, both murine and human IL-7 are capable of binding to receptors on cells of the opposite species.

Further characterization of TSLPR-mediated binding and signal transduction was done by using the BAF/B03 cell line, which expresses endogenous TSLPR and γc, but no IL-7Rα. This line binds TSLP with low affinity only, does not bind IL-7, and is incapable of proliferating in response to either cytokine. When a stable transfectant of this line was generated that expressed the IL-7Rα subunit as well, the cells were now found to bind both IL-7 and TSLP with high affinity and to proliferate in response to these cytokines. This result indicated that, at a minimum, the TSLPR and IL-7Rα subunits were required to generate a functional TSLPRcomplex. This conclusion was further substantiated by the observation that in Whitlock-Witte cultures established from IL-$7R\alpha^{-/-}$ mice, TSLP was incapable of stimulating cell growth. TSLP did show activity in this culture system, however. When combined with SCF, TSLP stimulated growth of wild-type B220⁺ cells that were absent in cultures grown in SCF alone.

Signal transduction in the IL-7 receptor complex requires the γc chain, first described for the IL-2 receptor complex (29, 33). The possibility that the γc might be involved in TSLP signal transduction was tested functionally by studying the in vitro proliferative response of thy-

mocytes and pre-B cells from $\gamma c^{-/-}$ mice to IL-7 and TSLP. In each case, there was an insignificant response of $\gamma c^{-/-}$ cells to IL-7, as expected; however, $\gamma c^{-/-}$ cells were still competent to respond to TSLP. In total, these data indicate that high affinity binding and biological activity of TSLP require the IL-7Rα chain, but do not require the γc chain of the IL-2R complex.

The relationship of TSLP and IL-7 to their cellular receptors is analogous to the IL-13–IL-4 cytokine–receptor system in which IL-13–mediated signaling through the IL-13R utilizes the IL-4Rα chain but not the γc chain (34, 35). Interestingly, both IL-7 and TSLP have overlapping biological activities as well as unique properties. Both IL-7 and TSLP promote growth of B cell and T cell precursors; however, the unique biological responses generated through binding of TSLP to the TSLPR–IL-7Rα complex have yet to be well understood. A recent report describes the ability of TSLP and IL-7 to induce tyrosine phosphorylation of the signal transducer and activator of transcription (Stat)5 transcription factor. TSLP did not induce activation of Janus kinase 1 and Janus kinase 3, whereas IL-7–mediated signaling did so in the same cell line (36). This evidence indicates that TSLP will have biological effects that are not common to IL-7, and these remain to be elucidated in detail.

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